

Alkannin attenuates amyloid β aggregation and Alzheimer's disease pathology

Toru Hosoi^{1*#}, Kyosuke Yazawa^{2#}, Michihiro Imada², Akari Tawara², Chihiro Tohda⁴,
Yasuyuki Nomura³, Koichiro Ozawa^{2*}

¹Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences,
Sanyo-Onoda City University, 1-1-1 Daigaku-dori, Sanyo Onoda City, Yamaguchi
756-0884, Japan

²Department of Pharmacotherapy, Graduate School of Biomedical and Health Sciences,
Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

³Department of Pharmacology, Kurume University School of Medicine, 67 Asahi-machi,
Kurume, Fukuoka 830-0011, Japan.

⁴Section of Neuromedical Science, Division of Bioscience, Institute of Natural
Medicine, University of Toyama, Toyama, Japan.

equal contribution to the present study

***corresponding author:**

Toru Hosoi¹ or Koichiro Ozawa²

e-mail: hosoi@rs.socu.ac.jp, ozawak@hiroshima-u.ac.jp

¹Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences,
Sanyo-Onoda City University, 1-1-1 Daigaku-dori, Sanyo Onoda City, Yamaguchi
756-0884, Japan

²Department of Pharmacotherapy, Graduate School of Biomedical and Health Sciences,
Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Competing Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Keywords: Alzheimer's disease, amyloid β , aggregation, chemical chaperone

Running title:

Alkannin attenuates amyloid β aggregation

The total number of manuscript pages: 29

Figures: 7

Tables: 0

Supplemental figures: 2

Total word count of the Abstract: 179

Total word count of the Introduction: 469

Total word count of the Discussion: 870

Abbreviations

α -LA, α -lactalbumin; Alzheimer's disease; A β , amyloid β ; A β_{1-42} , Amyloid β_{1-42} ; AD, APP, amyloid precursor protein; CD, circular dichroism; *C. elegans*, *Caenorhabditis elegans*; CI, chemotaxis index; DMSO, dimethylsulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LDH, lactate dehydrogenase; NGM, nematode growth medium; ThT, thioflavin T

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease that is accompanied by memory decline and cognitive dysfunction. Aggregated amyloid β formation and accumulation may be one of the underlying mechanisms of the pathophysiology of AD. Therefore, compounds that can inhibit amyloid β aggregation may be useful for treatment. Based on this hypothesis, we screened plant compounds used in Kampo medicine for chemical chaperone activity and identified that alkannin had this property. Further analysis indicated that alkannin could inhibit amyloid β aggregation. Importantly, we also found that alkannin inhibited amyloid β aggregation after aggregates had already formed. Through the analysis of circular dichroism spectra, alkannin was found to inhibit β -sheet structure formation, which is an aggregation-prone toxic structure. Furthermore, alkannin attenuated amyloid β -induced neuronal cell death in PC12 cells, ameliorated amyloid β aggregation in the AD model of *Caenorhabditis elegans* (*C. elegans*), and inhibited chemotaxis observed in AD *C. elegans*, suggesting that alkannin could potentially inhibit neurodegeneration *in vivo*. Overall, these results suggest that alkannin may have novel pharmacological properties for inhibiting amyloid β aggregation and neuronal cell death in AD.

Significance statement

Aggregated amyloid β formation and accumulation is one of the underlying mechanisms of the pathophysiology of Alzheimer's disease. We found that alkannin had chemical chaperone activity, which can inhibit β -sheet structure formation of amyloid β and its aggregation, neuronal cell death, and Alzheimer's disease phenotype in *C. elegans*. Overall, alkannin may have novel pharmacological properties for inhibiting amyloid β aggregation and neuronal cell death in Alzheimer's disease.

Introduction

Amyloid β protein is produced by the cleavage of amyloid precursor protein (APP), a type I transmembrane glycoprotein. APP is cleaved by two enzymes, β -secretase and γ -secretase, which produce amyloid β protein (Selkoe, 2011). Amyloid β protein is prone to aggregation, and while monomers of amyloid β protein do not induce neuronal cell death, the aggregation of amyloid β protein can (Pike et al., 1993; Lorenzo et al., 1994). It has been hypothesized that the pathophysiology of Alzheimer's disease (AD) involves the accumulation of aggregated amyloid β protein in the brain, possibly in senile plaque, which is observed in the AD brain. In several mouse models, the induced accumulation of amyloid β protein has been found to cause neurodegeneration (Götz et al., 2018). Furthermore, evidence suggests that aggregated amyloid β protein may act as a transmitter (Jaunmuktane et al., 2015; Meyer-Luehmann et al., 2006). Overall, these reports suggest that aggregated amyloid β protein accumulation and transmission may play a key role in the pathogenesis of AD.

The inhibition of amyloid β protein aggregation may therefore be a key strategy for the treatment of AD. Several potential methods for reducing the accumulation of aggregated amyloid β proteins have been presented. One such method is the use of chemical chaperones (Cohen et al., 2003). Chemical chaperones are compounds of low molecular weight that can inhibit protein aggregation (Perlmutter et al., 2002). Several reports have indicated the use of chemical chaperones in protein aggregation-mediated diseases such as cystic fibrosis (Hanrahan et al., 2013), diabetes (Ozcan et al., 2006), and epilepsy (Yokoi et al., 2015). Previous studies have also reported that endoplasmic reticulum stress is linked to leptin resistance in obesity, which can be ameliorated by chemical chaperones (Hosoi et al., 2014; Ozcan et al., 2009;

Hosoi et al., 2008). Interestingly, the amyloid-binding dye thioflavin T (ThT) has been found to extend the lifespan and reduce β -amyloid-associated toxicity in *C. elegans* (Alavez et al., 2011). Furthermore, Congo red, a histological dye, has been shown to inhibit the fibril formation activity of amyloid β protein and thus reduce associated toxicity against neuronal cells (Lorenzo et al., 1994). However, ThT and Congo red are mainly used to stain aggregated β -amyloid for diagnosis and may not be usable in medicine. Therefore, in the present study, we aimed to identify compounds that could ameliorate amyloid β protein aggregation from the library of plant compounds used in Kampo medicine, a traditional Chinese medicine. We reasoned that, since the compounds derived from Kampo medicine have been used for hundreds of years, they would be safe. Based on this screening, we found that alkannin has chemical chaperone activity and can thereby attenuate amyloid β protein aggregation and ameliorate AD pathology. In the present study, we therefore investigated the pharmacological effects of alkannin on amyloid β aggregation and neuronal cell death.

Materials and Methods

Materials

The human amyloid β_{1-42} peptide was obtained from the Peptide Institute (Osaka, Japan). The library of plant compounds was obtained from the Institute of Natural Medicine, University of Toyama. Each compound was dissolved at 10 mM in dimethylsulfoxide (DMSO) and stored at -20°C until use.

Measurement of chaperone activity

Chaperone activity measurements were performed as previously described (Huang et al., 2000; Li et al., 2001; Kubota et al., 2006). The assay was based on measuring α -LA aggregation, which was analyzed by measuring optical density at 488 nm to detect turbidity using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Preparation of amyloid β

$\text{A}\beta_{1-42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at 1 mg/mL and sonicated in an ice-cooled water bath for 4 min. The samples were then aliquoted in a tube, dried under vacuum, and stored at -20°C until use.

Measurement of amyloid β aggregation using thioflavin T

Amyloid β aggregation was measured based on previous reports, with slight modifications (Feng et al., 2009). HFIP-treated $\text{A}\beta_{1-42}$ was dissolved in DMSO at 1

mg/mL and then diluted to 10 μ M with phosphate-buffered saline. The compounds were added to the A β ₁₋₄₂ solution and incubated at 37°C for the indicated times. After incubation, 180 μ L of 5 μ M ThT solution was added to 20 μ L of the A β ₁₋₄₂ solution and the fluorescence of ex 450 nm/em and 480 nm was measured. The ThT solution (5 μ M) was prepared using 50 mM phosphate buffer (pH 6.5).

Dot blotting

Amyloid β aggregation was measured based on previous reports, with slight modifications (Izuo et al., 2012). HFIP-treated A β ₁₋₄₂ was dissolved in DMSO at 1 μ M and then diluted to 20 μ M with phosphate-buffered saline. Alkannin was added to the solution to a final concentration of 10 μ M and incubated at 37°C for 8 h. Control samples were treated with DMSO to a final concentration of 0.1%. The samples were then applied to nitrocellulose membranes; i.e., 2 μ L for 6E10 and 5 μ L for 11A1. After 10 min, the membranes were blocked by 2.5% skim milk in Tris-buffered saline containing 0.1% Tween-20. Then, the membranes were probed with one of the primary antibodies, anti-A β ₁₋₁₇ (6E10, 1 μ M) or anti-E22P-A β ₁₀₋₃₅ (11A1, 1 μ M) overnight at 4°C followed by incubation with the secondary antibody (1 h at room temperature). The intensities of the dots were quantified using Image J software.

Cell culture

PC12 cells were maintained in an RPMI medium with 5% (v/v) heat-inactivated fetal calf serum, 10% (v/v) heat-inactivated horse serum, 100 units/mL penicillin G, and 100 mg/mL streptomycin. Cultured cells were maintained at 37°C in 5% CO₂/95% air. PC12 cells were cultured on poly D-lysine-coated plates for the assay. Three days

before A β_{1-42} stimulation, the cultured medium was replaced with a neurobasal medium containing 2% B-27 supplement and 0.5 mM L-glutamine. On the day of A β_{1-42} stimulation, the cultured medium was replaced with a neurobasal medium containing 2% B-27 supplement without anti-oxidant and 0.5 mM L-glutamine. For A β_{1-42} stimulation, amyloid β_{1-42} peptide was dissolved in 0.02% NH₃ (200 μ M) and added to the PC12 cells to obtain the final concentration indicated in the results section.

Measurement of cell death using lactate dehydrogenase assay

Cell death was analyzed by measuring the amount of LDH released into the medium. The LDH assay was performed using a cytotoxicity detection kit (Roche Molecular Biochemical, Basel, Switzerland) according to the manufacturer's instructions. LDH activity was measured at an optimal density of 492 nm. The percentage of cell death was calculated by measuring the ratio of LDH activity in the culture medium to the total LDH activity (i.e., [extracellular LDH] / [extracellular LDH + cellular LDH]).

Measurement of amyloid β secondary structure by analyzing circular dichroism spectra

A 200 μ M A β_{1-42} stock solution (in 0.02% NH₃) was dissolved in a 50 mM potassium phosphate buffer to obtain a final concentration of 10 μ M of A β_{1-42} . Concurrently, alkannin was added to the solution to a final concentration of 10 μ M. Control samples were treated with DMSO to a final concentration of 0.1%. The samples were then incubated at 37°C for 24 h to form the secondary structure of A β_{1-42} . The CD spectra were measured using a spectropolarimeter J720 (JASCO, Japan).

Measurement of amyloid β fibrils using scanning electron microscope

HFIP-treated A β ₁₋₄₂ was dissolved in DMSO, sonicated, and then diluted to 10 μ M with phosphate-buffered saline. The samples were then incubated at 37°C for 48 h. A scanning electron microscope (SEM) analysis was performed at the Hiroshima University of Imaging Platform, Japan.

Maintenance of *Caenorhabditis elegans*

The N2 control and CL2122, CL2355, and CL4176 worm strains were maintained on nematode growth medium (NGM) plates with OP50 as a food source. These strains were obtained from the *Caenorhabditis* Genetics Center. For the experiments, the age of the *C. elegans* was synchronized by bleaching. Samples were then plated and OP50 bacteria were added after the eggs had hatched. They were then grown at 16–25°C until adulthood. 5-Fluoro-2'-deoxyuridine (FUdR) was added 72 h after synchronization.

Chemotaxis assay of *C. elegans*

A chemotaxis assay of *C. elegans* was performed 144 h after synchronization. Following several washes with M9 buffer, *C. elegans* was plated at the center of the NGM plate. For the assay plate, 1 μ L of 1% benzaldehyde (dissolved in ethanol) and 0.5 μ L of 1M sodium azide were added to the attractant side of the plate. Similarly, 1 μ L of ethanol and 0.5 μ L of 1M sodium azide were added on the opposite side of the plate for the control. The plate was then incubated at 23°C in a dark room for 4 h, after which the amount of *C. elegans* that had moved was counted and the chemotaxis index was calculated using the following formula: chemotaxis index (%) = [(number of

attractant side – number of control side) / number of *C. elegans*] \times 100.

Staining of amyloid β in *C. elegans*

After hatching, CL4176 cells were incubated at 16°C for 36 h, treated with alkannin (3 μ M), and then incubated at 25°C for another 36 h. After incubation, the worms were washed with M9 buffer and transferred to microfuge tubes. The worms were then fixed with 4% paraformaldehyde in M9 buffer (pH 7.4), incubated at 4°C for 24 h, and subsequently permeabilized with 5% 2-mercaptoethanol, 1% Triton X-100, and 125 mM Tris (pH 7.4), at 37°C for 24 h. After washing twice with M9 buffer, the worms were stained with 0.125% ThT (in 50% ethanol) for 2 min. The worms were then sequentially washed with 50%, 70%, 90%, 70%, and 50% ethanol for 2 min each. Finally, they were washed with M9 buffer containing 1% Triton X-100, and fluorescence images were acquired using a microscope (Keyence, BZ9000). For the data analysis, we used non-treatment AD model *C. elegans* (CL4176 strain) sample as control, and WT, DMSO control- or Alkannin-treated samples were divided by non-treatment AD sample (WT, DMSO control or Alkannin AD / non-treatment AD).

Statistics

The results are expressed as the mean \pm SD. Statistical analyses were performed using the paired *t*-test, Tukey-Kramer or Dunnett's test. We considered the data to be statistically significant, when P values were lower than 0.05.

Results

Identification of candidate compound from screening assay

A compound that could inhibit A β aggregation may be a candidate drug for the treatment of AD. To identify such a compound, we first performed a screening assay. We selected compounds derived from traditional Chinese medicine to create a library for the screening assay. The list of compounds used in the present study is shown in Supplementary Figure 1. We performed two different types of screening assays: a measurement of chemical chaperone activity and a measurement of amyloid β aggregation. The chaperone activity measurement was based on α -lactalbumin (α -LA) aggregation (Huang et al., 2000; Li et al., 2001; Kubota et al., 2006). We incubated the cells with or without each compound for 6 h at 37°C and measured the chemical chaperone activity. Using this assay, we identified three compounds (alkannin, coptisine chloride, and shikonin) that had chemical chaperone activity (Fig. 1A). Next, we screened amyloid β aggregation using a ThT assay (LeVine H 3rd et al., 1993). We incubated the cells with or without each compound for 7 h at 37°C and measured amyloid β aggregation using the ThT assay. From this assay, we found that five compounds, (alkannin, barbaloin, (z)-ligustilide, luteolin, and shikonin) had inhibitory effects on the aggregation of amyloid β (Fig. 1B). Based on these two assays, we found that only alkannin and shikonin had both chemical chaperone activity and inhibitory effects on amyloid β aggregation. Alkannin and shikonin are enantiomers of each other. In the present study, we focused on alkannin and analyzed the effect of alkannin on AD pathology in more detail.

Alkannin inhibits A β aggregation

To confirm the results of the screening assay, we performed a time-course analysis of A β aggregation using the ThT assay. Amyloid β_{1-42} (A β_{1-42}) (10 μ M) was incubated with or without alkannin (10 μ M) for 0, 6, 12, 24, and 48 h at 37°C, and A β_{1-42} aggregation was measured. A β_{1-42} alone increased aggregation in a time-dependent manner (Fig. 2B). However, the co-treatment of A β_{1-42} and alkannin dramatically attenuated A β_{1-42} aggregation at 6, 12, 24, and 48 h (Fig. 2B). Alkannin alone did not affect the ThT-induced O.D. signal, suggesting that alkannin may not directly affect the action of ThT. These results suggest that alkannin inhibits A β_{1-42} aggregation without secondary effects.

To determine whether alkannin could ameliorate preformed amyloid β aggregation, we treated amyloid β aggregates with alkannin. To achieve this, we preincubated A β_{1-42} for 6 or 24 h and observed a dramatic increase in A β_{1-42} aggregation (Fig. 2CD). After preincubating A β_{1-42} for 6 or 24 h, we then added alkannin and further incubated for 6–42 h and analyzed A β_{1-42} aggregation. To compare the effect of shikonin versus alkannin on A β_{1-42} aggregation, we next analyzed the effect of shikonin and alkannin on A β_{1-42} aggregation at 6, 12, and 24 h. As shown in Fig. 2E, we observed similar inhibitory action against A β_{1-42} aggregation (Fig. 2E). From these experiments, we found that A β_{1-42} aggregation was attenuated by alkannin (Fig. 2). These results indicate that alkannin can also ameliorate preformed A β aggregates, suggesting that alkannin may be beneficial for the treatment and prevention of AD.

Alkannin inhibits the formation of the β -sheet secondary structure of amyloid β

After finding that alkannin inhibited amyloid β aggregation, we next analyzed

whether alkannin affected the secondary structure of amyloid β . To analyze this, we performed circular dichroism (CD) spectral analysis. In general, the secondary structure of amyloid β is randomly coiled when it is in monomer formation, whereas it changes to a β -sheet structure during multimer formation and aggregation. We analyzed the CD spectra before and after incubation with $A\beta_{1-42}$ (10 μ M) at 37°C for 24 h. After incubating $A\beta_{1-42}$ for 24 h, we observed the typical CD spectra of the β -sheet structure (Fig. 3B panel of 24h). We found that these typical CD spectra were ameliorated by 10 μ M of alkannin treatment (Fig. 3B). In contrast, the CD spectra were not affected by alkannin treatment alone, suggesting that alkannin itself does not affect the CD spectra (Fig. 3AB). These results suggest that alkannin may affect conformational structural changes in amyloid β , thereby inhibiting the β -sheet secondary structure of amyloid β .

Alkannin moderately attenuated amyloid β fibril formation

Since we found that alkannin could inhibit the β -sheet secondary structure of amyloid β and subsequent formation of aggregation, we presumed that alkannin could also attenuate amyloid β fibril formation. To analyze this, we performed an electron microscopy analysis. $A\beta_{1-42}$ (10 μ M) was incubated for 48 h at 37°C with or without alkannin (10 μ M), and amyloid β fibril formation was analyzed. Amyloid β fibril formation was detected by electron microscopy analysis, which showed the width of the fibril formed was moderately reduced by alkannin (Fig. 3C). These results indicate that alkannin may also moderately inhibit amyloid β fibril formation.

Alkannin inhibited toxic conformer of amyloid β

As we found that alkannin can inhibit aggregation of amyloid β , we next investigated whether alkannin can inhibit toxic conformer of amyloid β . To this end, we had done dot blotting of amyloid β using 11A1 antibody, which can specifically bind with toxic conformer of amyloid β (Izuo et al., 2012). Amyloid β_{1-42} was incubated for 8 h at 37 °C with or without alkannin. We observed increase in dot blotting signal of 11A1 antibody, when incubated for 8 h (Fig. 4). On the other hand, we observed attenuation of the signal when we treated with alkannin. No difference was observed when we used anti-A β 1-17 antibody (6E10) antibody (Fig. 4). These results suggest that alkannin may inhibit toxic conformer of Amyloid β .

Alkannin has a neuroprotective effect on amyloid β -induced cell death

Aggregated amyloid β is toxic to neurons (Pike et al., 1993; Lorenzo et al., 1994). Since we found that alkannin could reduce amyloid β aggregation, we next investigated whether alkannin had a neuroprotective effect on amyloid β -induced cell death. To test this, we treated PC12 neuronal cells with alkannin and analyzed A β_{1-42} -induced cell death. PC12 cells were treated with alkannin (0.1–1 μ M) and A β_{1-42} (5 μ M) for 48 h, and cell death was analyzed by measuring lactate dehydrogenase (LDH). We observed that A β_{1-42} caused cell death from 13.0% to 25.6%, and this effect was reduced to 17.8 % by alkannin treatment (Fig. 4). An enantiomer of alkannin, shikonin has been reported to protect against amyloid β -induced cell death (Tong et al., 2018). Therefore, to compare the neuroprotective action of these compounds, we also analyzed the effect of shikonin on A β_{1-42} (5 μ M)-induced cell death. As shown in Fig 5, shikonin similarly inhibited A β_{1-42} (5 μ M)-induced cell death (Fig 5). These results suggest alkannin can have a protective effect on amyloid β -induced neuronal cell death.

Alkannin attenuates chemotaxis in an AD *C. elegans* model

Since we found that alkannin attenuates amyloid β -induced neuronal cell death, we next investigated whether alkannin could ameliorate AD pathology *in vivo*. In the present study, we used an AD model of *C. elegans*. The worm strain CL2355 has a transgene that expresses human amyloid β peptide, which shows defective in chemotaxis associated with learning (Wu et al., 2006). The worm has a temperature-sensitive transgene, which expresses human amyloid β peptide when the culture conditions are shifted from 16 °C to 25 °C. Therefore, CL2355 was maintained at 16 °C during synchronization and changed to 25 °C 36 h after the synchronization. A control strain of *C. elegans* (CL2122) was maintained at 25°C. The basal level of the chemotaxis index (CI) of CL2122 was approximately 60 %. On the other hand, the chemotaxis index (CI) of the AD *C. elegans* (CL2355) was approximately 35% (Fig. 6), indicating an AD phenotype. We next investigated the effect of alkannin on the AD phenotype observed in CL2355. Alkannin (0.3, 1, and 3 μ M) was added 36 h after synchronization and chemotaxis was analyzed 72 h after drug application. We observed that the chemotaxis index was improved by alkannin in CL2355, the AD model of *C. elegans* (Fig. 6), while alkannin did not affect the chemotaxis index in the wild-type control *C. elegans* (CL2122) (Supplementary Figure 2), suggesting that the effect of alkannin was specific to the AD model of *C. elegans*. These results suggest that alkannin can ameliorate AD pathology.

Alkannin attenuates amyloid β aggregation in *C. elegans* *in vivo*

Since we found that alkannin could attenuate AD pathology in *C. elegans*, we next

investigated whether alkannin could ameliorate amyloid β aggregation *in vivo*. To achieve this, we treated an AD model of *C. elegans* (CL4176) with alkannin and analyzed amyloid β aggregation by measuring ThT-induced fluorescence. CL4176 is a temperature-sensitive A β_{1-42} transgene transgenic worm. In wild-type control worms (N2), which do not express human A β_{1-42} , the ThT fluorescence signal was almost undetectable (Fig. 6). In contrast, we detected a dramatic 7.9-fold increase in ThT fluorescence in CL4176, the AD model worms (Fig. 7). These results suggest that ThT can specifically detect aggregated A β_{1-42} in CL4176 worms. We next investigated whether A β_{1-42} aggregation in CL4176 was attenuated by alkannin. As shown in Fig. 7, we observed a decrease in the ThT fluorescence signal in alkannin-treated AD worms. These results suggest that alkannin can attenuate amyloid β aggregation *in vivo*.

Discussion

Several efforts have been challenged to identify compounds, which can ameliorate aggregation of amyloid β peptide (Giorgetti et al., 2018; Pagano et al., 2020). Among these compounds, dye, such as Congo red has been reported to ameliorate aggregation of amyloid β peptide. Alkannin is also a natural dye used for food coloring. However, currently, the relation between Congo red and alkannin against amyloid β aggregation are unknown. Alkannin is isolated from the roots of *Alkanna tinctoria*, a borage family compound, and has been used as a medicine for centuries (Papageorgiou et al., 1978). Alkannin has been reported to have anti-tumor activity (Zhang et al., 2018) and wound healing properties (Papageorgiou et al., 1978; Papageorgiou et al., 1999). However, the molecular mechanisms underlying its action on wound-healing properties are not well understood. Induction of protein synthesis and protein folding is required during wound healing (Bachar-Wikstrom et al., 2021; Schürmann et al., 2014). When unfolded proteins accumulate, endoplasmic reticulum stress is activated (Walter et al., 2011). Interestingly, endoplasmic reticulum stress may be involved in wound healing and the activation of protein folding by the chemical chaperone 4-PBA improved it (Bachar-Wikstrom et al., 2021; Schürmann et al., 2014). In the present study, we found that alkannin has chemical chaperone activity (Fig. 1A). Therefore, one of the mechanisms by which alkannin may contribute to wound healing may be mediated through this chemical chaperone activity.

In the present study, we found that alkannin may have a pharmacological mechanism of action for inhibiting formation of β sheet structure of amyloid β , thereby inhibit amyloid β aggregation and AD pathology. We performed ThT fluorescence assay for the measurement of amyloid β aggregation. However, we could not deny the possibility

that alkannin may replace ThT bound to the amyloid β aggregates/fibrils, which may be the limitation of this assay. Therefore, we had done different assay to confirm the results. We had done CD spectra analysis and found that alkannin reduced formation of β -sheet formation of amyloid β . As the formation of β -sheet structure is an essential step for the formation of aggregates, we think alkannin may indeed reduce aggregate of amyloid β .

It is reported that aggregated amyloid β is toxic to neurons (Pike et al, 1993; Lorenzo et al., 1994). Therefore, we assume that amyloid β -induced neuronal cell death of the present condition may be mediated through aggregated amyloid β . As we observed that alkannin reduced amyloid β aggregation, it is possible that inhibitory action of alkannin on amyloid β -induced cell death may be due to its inhibitory action against amyloid β aggregation. As we also observed that alkannin reduced toxic conformer of amyloid β , it is possible that alkannin's effect in inhibiting neuronal cell death may be due to the inhibitory effect against formation of toxic conformer of amyloid β . Alkannin has been reported to suppress NF- κ B signaling in LPS-induced lung injury (Li et al., 2019). Amyloid β -induced inflammation will be observed in brain glial cells, such as microglia or astrocytes (Kaur et al., 2019). Therefore, in addition to neuronal cells, alkannin may also affect glial inflammatory condition elicited by amyloid β . Amyloid β peptide accumulates at extracellular space (Marksteiner et al., 2008) and forms aggregate. As we found that alkannin can inhibit aggregation of amyloid β peptide, it is possible that alkannin inhibit aggregation of amyloid β at extracellular space. On the other hand, amyloid β peptide was also suggested to accumulate at intracellular level (LaFerla et al., 2007). It is possible that alkannin may penetrate through plasma membrane and affect amyloid β peptide aggregation as shikonin does

(Tong et al., 2018).

To analyze the observed effects *in vivo*, we performed chemotaxis assay in the transgenic *C. elegans* model of AD and found that alkannin recovered its chemotaxis. Considering this model, although the change in chemotaxis is a known phenotype, the causal relationship between reduced chemotaxis and amyloid β aggregation was not established. Therefore, we could not conclude that reduced chemotaxis was due to the reduced aggregation of amyloid β . On the other hand, using ThT analysis, we found that amyloid β was aggregated in this model and the aggregation was reduced by alkannin (Fig. 6). Therefore, it is possible that reduced aggregation of amyloid β by alkannin may contribute to the recovery of the chemotaxis in this model. Future analysis may require to investigate these *in vivo* effects.

AD is a progressive neurodegenerative disease that is active many years before the symptoms of dementia appear. Therefore, drugs that are safe and can be administered over a long period are required. Alkannin has been used as a food additive in New Zealand and Australia (Awuchi et al., 2020), suggesting that the compound is safe to consume. Moreover, alkannin has been used for many decades as a Kampo medicine. Therefore, alkannin may be a safe drug candidate for the treatment of AD. However, future analyses are required to investigate these possibilities.

In the present study, through a screening assay, we found that the compound alkannin could inhibit amyloid β aggregation. Further analyses revealed that alkannin has a neuroprotective effect against AD. Thus, the results support the hypothesis that amyloid β is actively involved in AD, and this compound may be useful for the treatment of AD.

Acknowledgments

SEM analysis was performed at the Hiroshima University of Imaging Platform supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. We appreciate Dr. Misako Imai-Takemoto for her kind support in the analysis of the electron microscope.

Authorship Contributions

Participated in research design: Hosoi, Ozawa, Tohda, Nomura

Conducted experiments: Yazawa, Imada, Tawara

Performed data analysis: Yazawa, Imada, Tawara

Wrote or contributed to the writing of the manuscript: Hosoi

References

- Awuchi CG, Twinomuhwezi H, Igwe VS, and Amagwula IO (2020). Food Additives and Food Preservatives for Domestic and Industrial Food Applications. *Journal of Animal Health*, 2(1), 1–16.
- Alavez S, Vantipalli MC, Zucker DJ, Klang IM, and Lithgow GJ. (2011) Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature* **472**: 226-9.
- Bachar-Wikstrom E, Manchanda M, Bansal R, Karlsson M, Kelly-Pettersson P, Sköldenberg O, and Wikstrom JD. (2021) Endoplasmic reticulum stress in human chronic wound healing: Rescue by 4-phenylbutyrate. *Int Wound J*. **18**: 49-61.
- Cohen FE, and Kelly JW. (2003) Therapeutic approaches to protein-misfolding diseases. *Nature* **426**: 905-909.
- Feng Y, Wang XP, Yang SG, Wang YJ, Zhang X, Du XT, Sun XX, Zhao M, Huang L, and Liu RT. (2009) Resveratrol inhibits beta-amyloid oligomeric cytotoxicity but does not prevent oligomer formation. *Neurotoxicology* **30**: 986-995.
- Giorgetti S, Greco C, Tortora P, and Aprile FA. (2018) Targeting Amyloid Aggregation: An Overview of Strategies and Mechanisms. *Int J Mol Sci*. **19**: 2677.
- Götz J, Bodea LG, and Goedert M. (2018) Rodent models for Alzheimer disease. *Nat Rev Neurosci*. **19**: 583-598.
- Hanrahan JW, Sampson HM, and Thomas DY. (2013) Novel pharmacological strategies to treat cystic fibrosis. *Trends Pharmacol Sci*. **34**: 119-125.
- Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, Yoshii M, and Ozawa K. (2008) Endoplasmic reticulum stress induces leptin resistance. *Mol Pharmacol*. **74**: 1610-1619.
- Hosoi T, Yamaguchi R, Noji K, Matsuo S, Baba S, Toyoda K, Suezawa T, Kayano T, Tanaka S, and Ozawa K. (2014) Flurbiprofen ameliorated obesity by attenuating leptin resistance induced by endoplasmic reticulum stress. *EMBO Mol Med*. **6**: 335-46.
- Huang G, Li ZY, and Zhou JM. (2000) Conformational specificity of trigger factor for

- the folding intermediates of alpha-lactalbumin. *Biochim Biophys Acta*. **1480**: 77-82.
- Izuo N, Kume T, Sato M, Murakami K, Irie K, Izumi Y, Akaike A. (2012) Toxicity in rat primary neurons through the cellular oxidative stress induced by the turn formation at positions 22 and 23 of A β 42. *ACS Chem Neurosci*. **3**: 674-81.
- Jaunmuktane Z, Mead S, Ellis M, Wadsworth JD, Nicoll AJ, Kenny J, Launchbury F, Linehan J, Richard-Loendt A, Walker AS, Rudge P, Collinge J, and Brandner S. (2015) Evidence for human transmission of amyloid- β pathology and cerebral amyloid angiopathy. *Nature* **525**: 247-50.
- Kaur D, Sharma V, and Deshmukh R. (2019) Activation of microglia and astrocytes: a roadway to neuroinflammation and Alzheimer's disease. *Inflammopharmacology* **27**: 663-677.
- Kubota K, Niinuma Y, Kaneko M, Okuma Y, Sugai M, Omura T, Uesugi M, Uehara T, Hosoi T, and Nomura Y. (2006) Suppressive effects of 4-phenylbutyrate on the aggregation of Pael receptors and endoplasmic reticulum stress. *J Neurochem*. **97**: 1259-68.
- LeVine H 3rd. (1993) Thioflavine T interaction with synthetic Alzheimer's disease β -amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci*. **2**: 404-410.
- LaFerla FM, Green KN, and Oddo S. (2007) Intracellular amyloid- β in Alzheimer's disease. *Nat Rev Neurosci*. **8**: 499-509.
- Li B, Lin Q, Hou Q, Yin C, Zhang L, and Li Y. (2019) Alkannin attenuates lipopolysaccharide-induced lung injury in mice via Rho/ROCK/NF- κ B pathway. *J Biochem Mol Toxicol*. **33**: e22323.
- Li J, Zhang S, and Wang C. (2001) Only the reduced conformer of α -lactalbumin is inducible to aggregation by protein aggregates. *J Biochem*. **129**: 821-826.
- Lorenzo A, and Yankner BA (1994) β -amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc Natl Acad Sci USA* **91**:12243–12247.
- Marksteiner J, Humpel C. (2008) β -amyloid expression, release and extracellular deposition in aged rat brain slices. *Mol Psychiatry***13**: 939-52.

- Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret JM, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, and Jucker M. (2006) Exogenous induction of cerebral β -amyloidogenesis is governed by agent and host. *Science* **313**: 1781-1784.
- Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, and Hotamisligil GS. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313**: 1137-1140.
- Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, Myers MG Jr, and Ozcan U. (2009) Endoplasmic reticulum stress plays a central role in development of leptin resistance. *Cell Metab.* **9**: 35-51.
- Pagano K, Tomaselli S, Molinari H, and Ragona L. (2020) Natural Compounds as Inhibitors of A β Peptide Aggregation: Chemical Requirements and Molecular Mechanisms. *Front Neurosci.* **14**: 619667.
- Papageorgiou VP. (1978) Wound healing properties of naphthaquinone pigments from *Alkanna tinctoria*. *Experientia* **34**: 1499-1501.
- Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, and Nicolaou KC. (1999) The Chemistry and Biology of Alkannin, Shikonin, and Related Naphthazarin *Natural Products Angew Chem Int Ed Engl.* **38**: 270-301.
- Perlmutter DH. (2002) Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. *Pediatr Res.* **52**: 832-836.
- Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, and Cotman CW (1993) Neurodegeneration induced by β -amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci* **13**:1676–1687.
- Selkoe DJ. (2011) Alzheimer's disease. *Cold Spring Harb Perspect Biol.* **3**: a004457.
- Schürmann C, Goren I, Linke A, Pfeilschifter J, and Frank S. (2014) Deregulated unfolded protein response in chronic wounds of diabetic ob/ob mice: a potential connection to inflammatory and angiogenic disorders in diabetes-impaired wound healing. *Biochem Biophys Res Commun.* **446**: 195-200.

- Tong Y, Bai L, Gong R, Chuan J, Duan X, and Zhu Y. (2018) Shikonin Protects PC12 Cells Against β -amyloid Peptide-Induced Cell Injury Through Antioxidant and Antiapoptotic Activities. *Sci Rep* **8**: 26.
- Walter P, and Ron D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334**: 1081-1086.
- Wu Y, Wu Z, Butko P, Christen Y, Lambert MP, Klein WL, Link CD, and Luo Y. (2006) Amyloid- β -induced pathological behaviors are suppressed by Ginkgo biloba extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans*. *J Neurosci*. **26**: 13102-13113.
- Yokoi N, Fukata Y, Kase D, Miyazaki T, Jaegle M, Ohkawa T, Takahashi N, Iwanari H, Mochizuki Y, Hamakubo T, Imoto K, Meijer D, Watanabe M, and Fukata M. (2015) Chemical corrector treatment ameliorates increased seizure susceptibility in a mouse model of familial epilepsy. *Nat Med*. **21**: 19-26.
- Zhang X, Cui JH, Meng QQ, Li SS, Zhou W, and Xiao S. (2018) Advance in Anti-tumor Mechanisms of Shikonin, Alkannin and their Derivatives. *Mini Rev Med Chem*. **18**: 164-172.

Footnotes

This work was supported by a Grant-in-Aid for the Cooperative Research Project from the Institute of Natural Medicine, University of Toyama in 2015 and 2016, JSPS KAKENHI [18KT0072], and the Takeda Science Foundation.

Conflict of interest

The authors have no conflicts of interest to declare.

Figures and Tables

Figure 1. Screening assay for the identification of candidate compounds used in Kampo medicine. (A) Screening of candidate compounds to determine which have chemical chaperone activity. The relative intensity of α -LA aggregation was analyzed by measuring optical density at 488 nm to detect turbidity after incubation at 37°C for 7 h. Mean \pm SD, n=3. (B) Screening of candidate compounds which could inhibit amyloid β aggregation. The relative intensity of amyloid β aggregation using ThT fluorescence intensity measured at an excitation wavelength of 450 nm and emission wavelength of 480 nm after incubating at 37°C for 6 h. Mean \pm SD, n=3. Candidate compounds were as follows: #5: alkannin, #15: barbaloin, #29: coptisine chloride, #65: (Z)-ligustilide, #69: luteolin, #91: shikonin. A list of all the compounds tested are shown in Supplementary Figure 1.

Figure 2 Alkannin inhibits the aggregation of amyloid β . (A) Chemical structure of alkannin. (B) A time-course analysis of the effect of alkannin on ThT fluorescence intensity was measured at 0, 6, 12, 24, and 48 h after incubating $A\beta_{1-42}$ (10 μ M) and alkannin (10 μ M). $A\beta_{1-42}$ alone dramatically increased ThT fluorescence intensity whereas $A\beta_{1-42}$ + alkannin did not. Alkannin alone did not affect ThT fluorescence intensity. Mean \pm SD, n=3. (C) Alkannin inhibited preformed amyloid β aggregates. Amyloid β was incubated for 6 h to form amyloid β aggregates in the absence of alkannin, after which the amyloid β aggregates were incubated with alkannin for the additional times and ThT fluorescence was measured. * P < 0.05, *** P < 0.001 vs cont (DMSO). Tukey-Kramer. Mean \pm SD, n=5 (D) Alkannin inhibited preformed amyloid β aggregates. $A\beta$ was incubated for 24 h to form amyloid β aggregates in the absence

of alkannin, after which the amyloid β aggregates were incubated with alkannin for the additional times. ThT fluorescence was measured after incubation. $*P < 0.05$, $**P < 0.01$ vs. control (DMSO). Tukey-Kramer. Mean \pm SD, $n=5$. (E) A time-course analysis of the effect of alkannin and shikonin on ThT fluorescence. The intensity was measured at 0, 6, 12, and 24 h after incubating $A\beta_{1-42}$ (10 μ M) and alkannin (10 μ M) or shikonin (10 μ M). Shikonin similarly attenuated ThT fluorescence intensity compared with alkannin. Mean \pm SD, $n=4$.

Figure 3. Alkannin attenuates the β -sheet structure and fibril formation of amyloid β . $A\beta_{1-42}$ (10 μ M) was incubated with or without alkannin (10 μ M) for 24 h at 37°C and CD spectra were analyzed. CD spectra of amyloid β and alkannin at (A) 0 h and (B) 24 h after incubation. Alkannin was found to attenuate the typical CD spectra signal of β -sheet structure formation of amyloid β . Mean \pm SD, $n=4$. (C) Alkannin attenuated amyloid β fibril formation. Scanning electron micrograph analysis of $A\beta_{1-42}$ (10 μ M) alone or $A\beta_{1-42}$ + alkannin (10 μ M) after incubation at 37°C for 48 h.

Figure 4. Alkannin attenuated formation of toxic conformer of amyloid β . (A) Amyloid β_{1-42} was incubated for 8 h with or without alkannin and analyzed toxic conformer of amyloid β using specific antibody 11A1. We observed inhibition of formation of the toxic conformer of amyloid by alkannin. On the other hand, similar signal was detected when we used anti- $A\beta_{1-17}$ antibody (6E10) antibody. (B) Densitometric analysis of the amyloid β dot blot analysis. Mean \pm SD, $n=5$. Tukey-Kramer $*p < 0.05$ vs control 8h.

Figure 5. Alkannin protects amyloid β -induced neuronal cell death. PC12 cells were treated with amyloid β (5 μ M) and alkannin (0.1, 0.3, and 1 μ M) or shikonin (0.1, 0.3, and 1 μ M). The percentage of cell death was measured by LDH assay after 48 h of incubation. Alkannin was found to attenuate amyloid β -induced neuronal cell death. Mean \pm SD, n=5. Dunnett's test compared with amyloid β -treatment alone was done. A β v.s A β + alkannin (P=0.14). A β v.s A β + shikonin (P=0.11).

Figure 6. Alkannin ameliorates AD and amyloid β aggregation in *C. elegans*. (A) Alkannin ameliorates chemotaxis index of AD in *C. elegans*. Treatment of CL2355 (AD model of *C. elegans*) with alkannin increased chemotaxis index (CI). CL2122 (normal control of *C. elegans*) was incubated at 25°C. CL2355 was incubated at 16°C initially; however, 36 h after hatching, the temperature was increased to 25°C. Concurrently, CL2355 was treated with alkannin (0.3, 1, 3 μ M). At 72 h after hatching, all the worms were treated with FUdR (0.6 mM) for sterilization. The chemotaxis assay was performed 144 h after hatching. CL2355 showed a reduced CI, which indicated AD, while treatment with alkannin showed a concentration-dependent increase in CI, which reached significance at 3 μ M. Mean \pm SD, n=6. * P < 0.05. Dunnett's test compared with AD model worms were done.

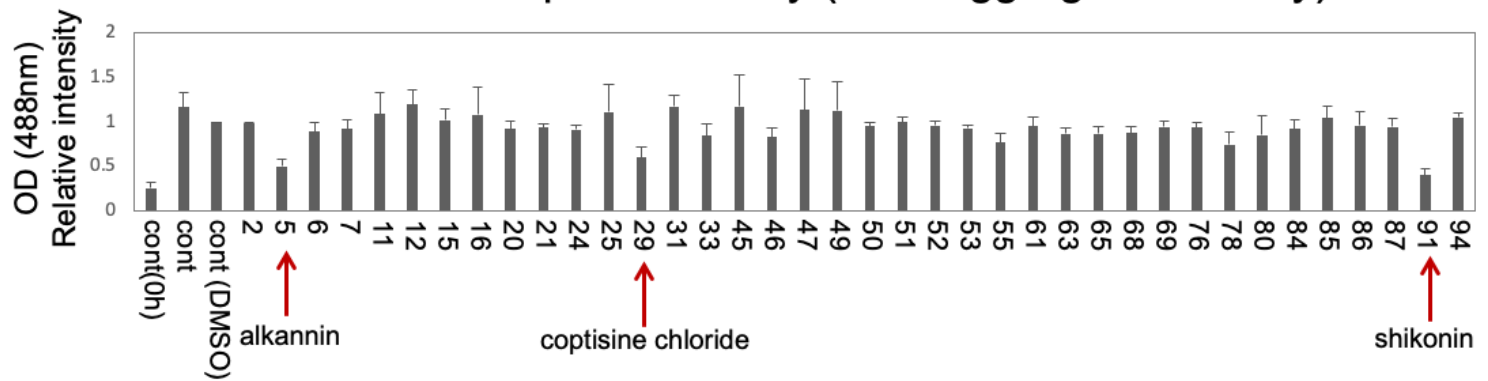
Figure 7. Reduced amyloid β aggregation in *C. elegans* treated with alkannin. CL4176 (AD model of *C. elegans*) was incubated at 16°C for 36 h. The worms were then treated with 3 μ M alkannin and incubated at 25°C for 36 h. After staining with thioflavin T, fluorescence images were acquired. (A) Fluorescence images of *C. elegans*. (B) Fluorescence intensity of *C. elegans*. Alkannin reduced aggregated

form of amyloid β in the AD model of *C. elegans*. Mean \pm SD, n=5. * $P < 0.05$.

Dunnett's test.

A

Chemical chaperon activity (α -LA aggregation assay)



B

A β aggregation (ThT assay)

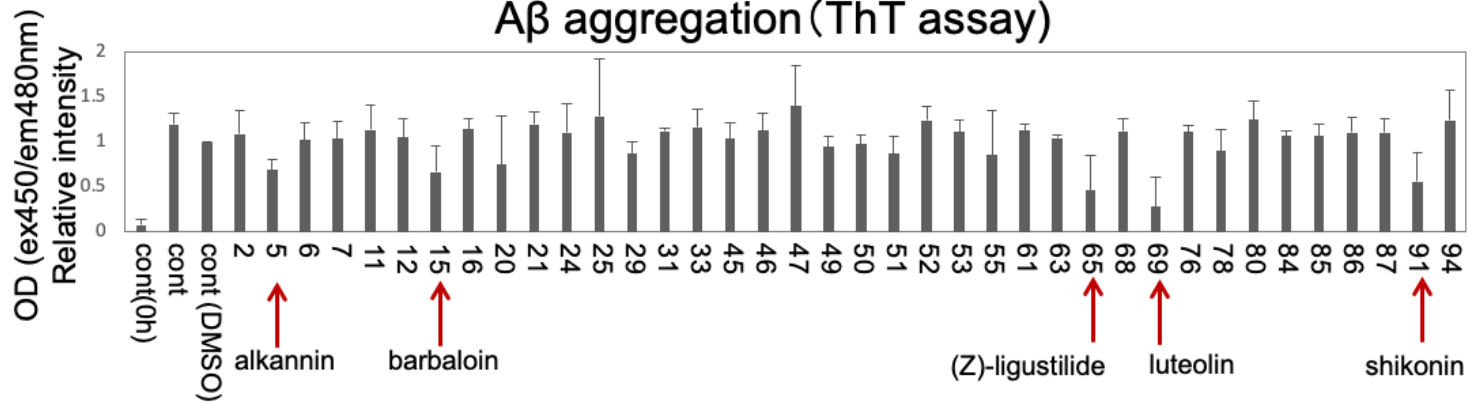


Figure 1

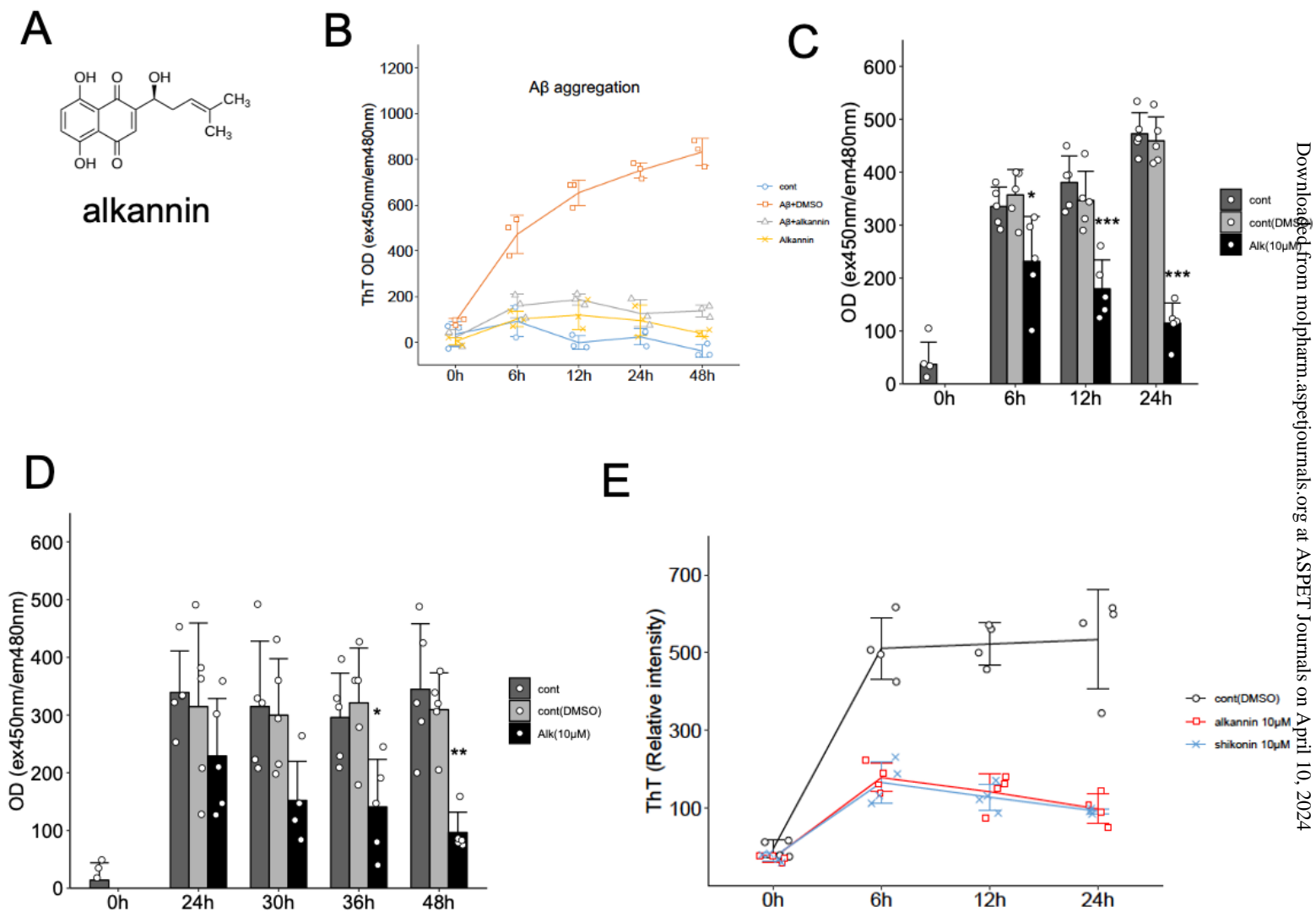


Figure 2

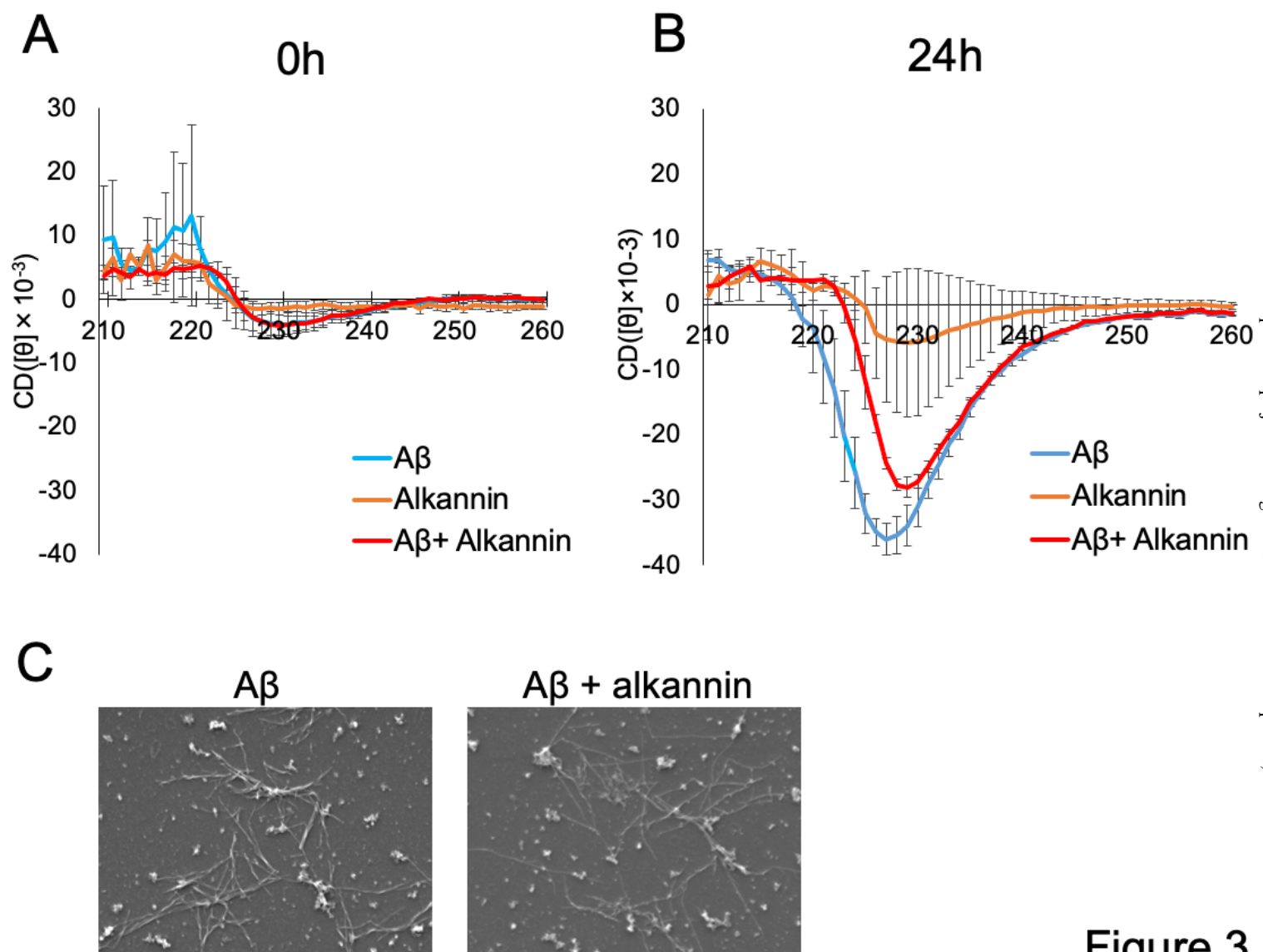


Figure 3

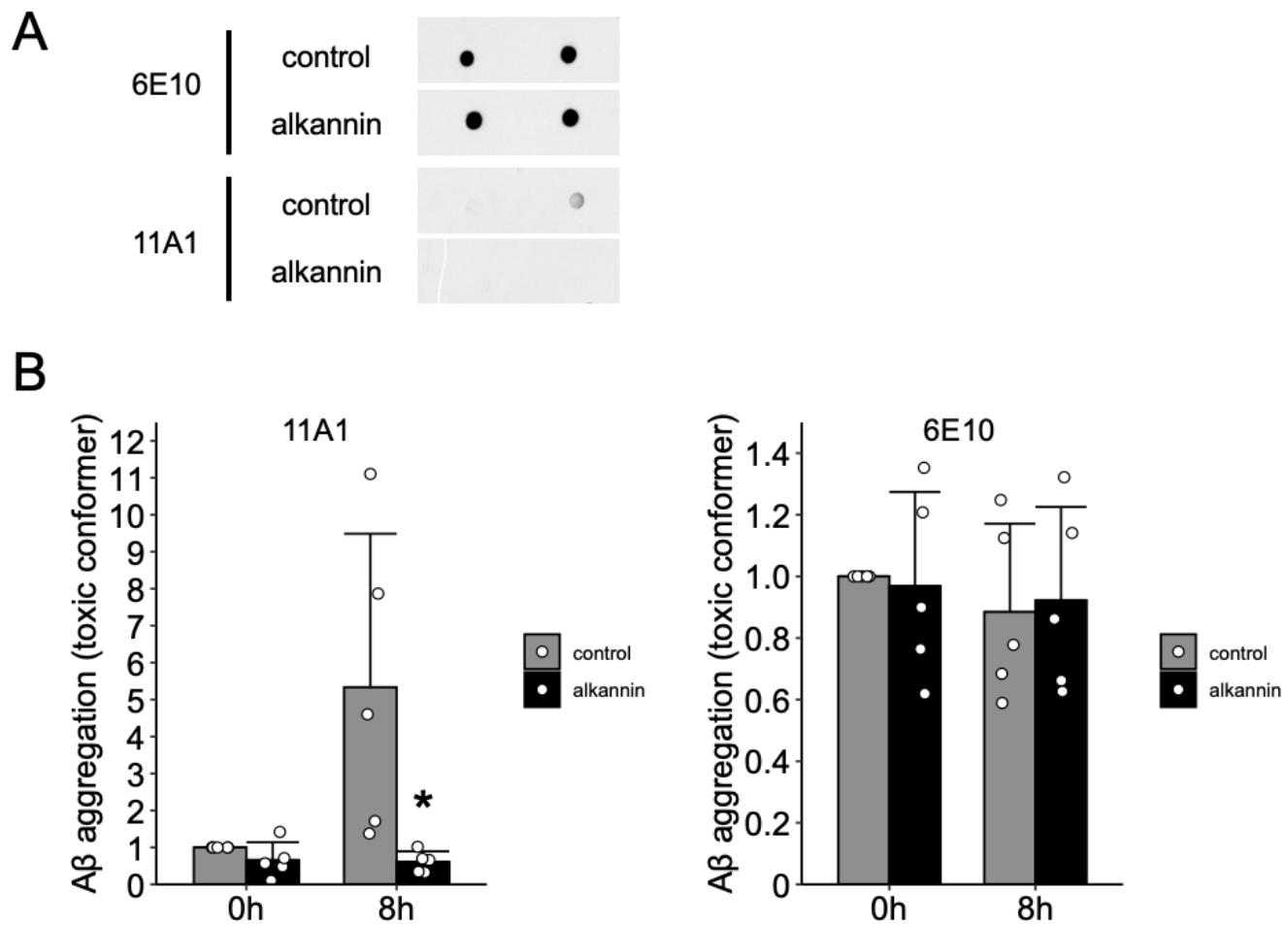


Figure 4

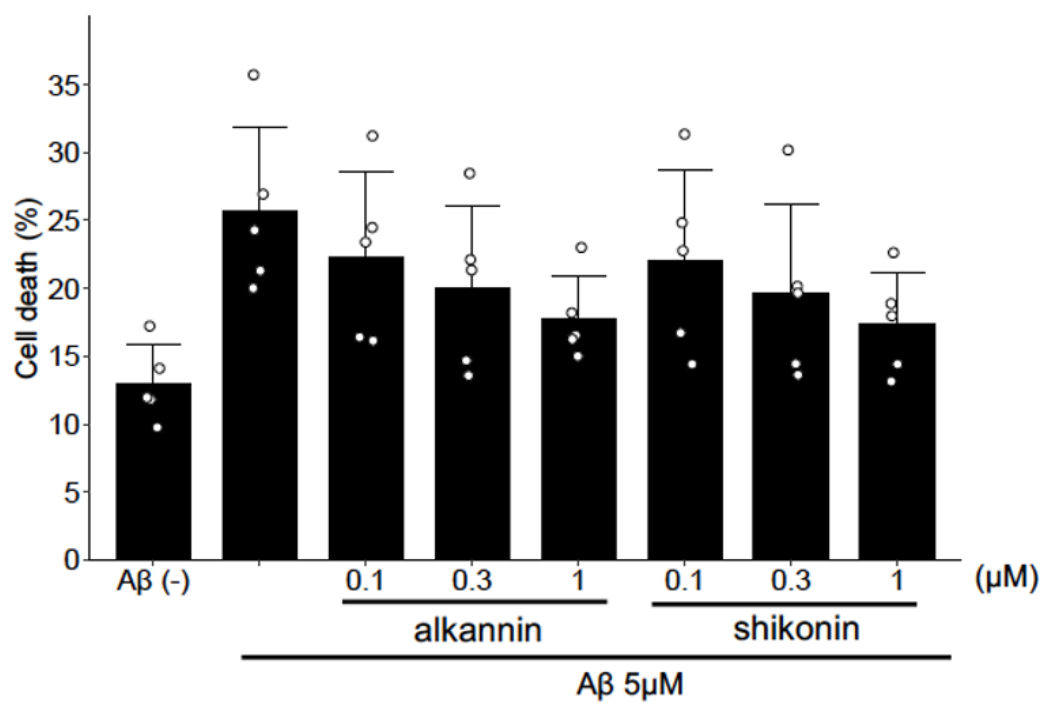


Figure 5

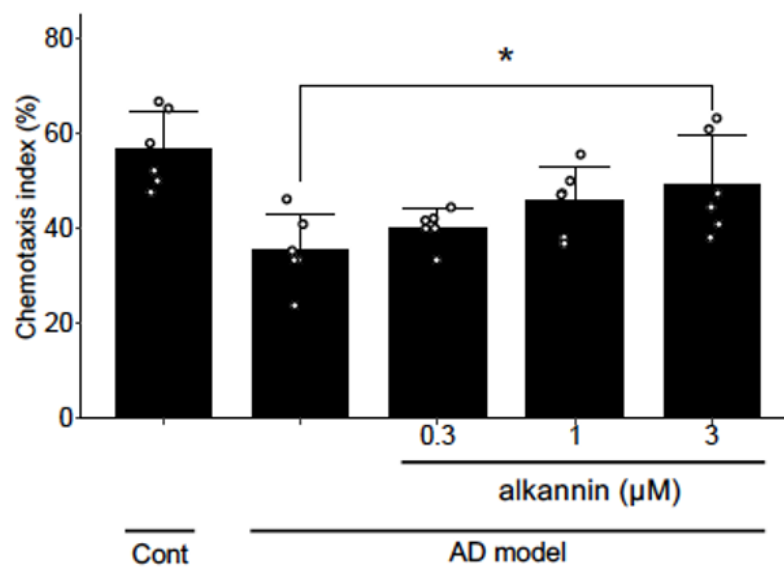


Figure 6

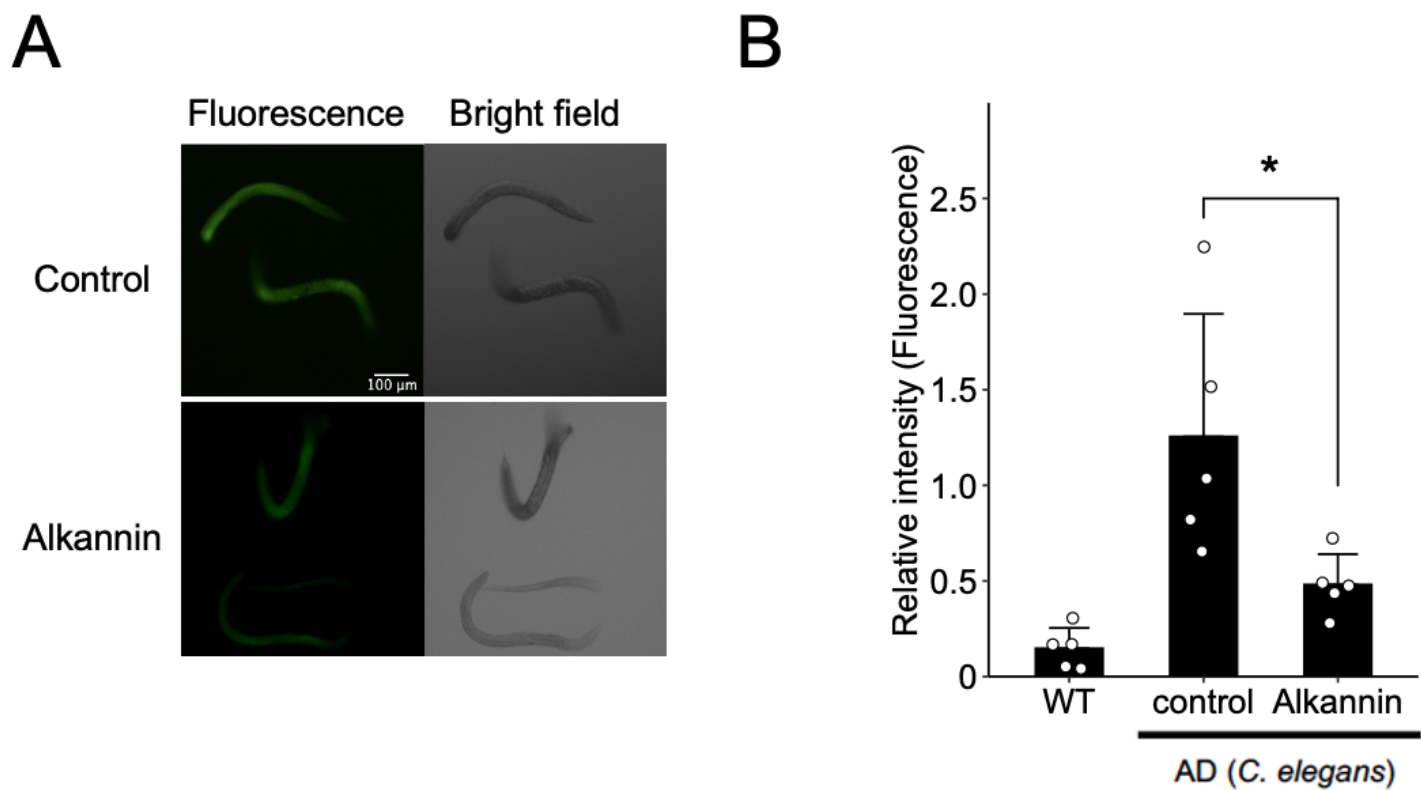


Figure 7